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Running title: Bid and NF-κB activation

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ABSTRACT

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease characterized by the loss of motoneurons in the spinal cord, brainstem and motor cortex. Mutations in the superoxide dismutase 1 (*SOD1*) gene represent a frequent genetic determinant and recapitulate a disease phenotype similar to ALS when expressed in mice. Previous studies using *SOD1*^{G93A} transgenic mice have suggested a paracrine mechanism of neuronal loss, in which cytokines and other toxic factors released from astroglia or microglia trigger motoneuron degeneration. Several pro-inflammatory cytokines activate death receptors and may downstream from this activate the Bcl-2 family protein, Bid. We here sought to investigate the role of Bid in astrocyte activation and non-cell autonomous motoneuron degeneration. We found that spinal cord Bid protein levels increased significantly during disease progression in *SOD1*^{G93A} mice. Subsequent experiments *in vitro* indicated that Bid was expressed at relatively low levels in motoneurons, but was enriched in astrocytes and microglia. Bid was strongly induced in astrocytes in response to pro-inflammatory cytokines or exposure to lipopolysaccharide. Experiments in *bid*-deficient astrocytes or astrocytes treated with a small molecule Bid inhibitor demonstrated that Bid was required for the efficient activation of transcription factor nuclear factor-κB in response to these pro-inflammatory stimuli. Finally, we found that conditioned medium from wild-type astrocytes, but not from *bid*-deficient astrocytes, was toxic when applied to primary motoneuron cultures. Collectively, our data demonstrate a new role for the Bcl-2 family protein Bid as a mediator of astrocyte activation during neuroinflammation, and suggest that Bid activation may contribute to non-cell autonomous motoneuron degeneration in ALS.

Highlights:

- Spinal cords from SOD^{G93A} transgenic mice exhibited markedly elevated levels of Bid protein
- Pro-inflammatory conditions increased Bid protein content in astrocytes but not in microglia
- Bid protein participated in the activation of nuclear factor-κB in astrocytes
- Bid plays a role in non-cell autonomous motoneuron death

Keywords Amyotrophic Lateral Sclerosis, Astrocyte, Motoneuron degeneration, *bid*, Bcl-2 family protein, $SOD1^{G93A}$, nuclear factor-κB.

Abbreviations: ALS, Amyotrophic Lateral Sclerosis; APAF1, Apoptotic protease activating factor 1, BH3-only protein, Bcl-2 homology domain 3-only protein; *bid*, BH3 interacting domain death agonist; GFAP, glial fibrillary acidic protein; Iba-1, ionized calcium-binding adapter molecule 1; IFNγ, interferon-γ; IKK, inhibitor of kappaB kinase; IL-1β, interleukin-1β; mtSOD1, mutant superoxide dismutase 1; NEMO, NF-kappa-B essential modifier; NF-κB, nuclear factor-κB; NOD1, nucleotide-binding oligomerization domain containing 1; SOD1, superoxide dismutase 1; PND, post-natal day; SMI-32, Sternberger monoclonal-incorporated antibody 32; tg, transgenic; wt, wild-type

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INTRODUCTION

Amyotrophic Lateral Sclerosis (ALS) is an adult-onset, progressive motoneuron disease characterized by degeneration of motoneurons in the motor cortex, brainstem and spinal cord ventral horns. Mutations in the copper/zinc superoxide dismutase gene (*SOD1*) account for approximately 20 - 25% of familial ALS patients (Deng et al., 1993; Rosen, 1993). Over-expression of the human familial ALS-linked $SOD1^{G93A}$ mutations in transgenic mouse models (*mtSOD1*) confer a phenotype similar to ALS patients with motor function deficits and a reduced lifespan (Gurney et al., 1994). Although the precise mechanisms of motoneuron degeneration in ALS remain largely unidentified, proteotoxicity, endoplasmic reticulum stress, glutamate excitotoxicity, oxidative stress, and activation of apoptosis have been shown to facilitate motoneuron death in *mtSOD1* mice and other ALS disease models (Bruijn et al., 2004; Pasinelli and Brown, 2006).

Apoptosis is a genetically controlled cell death process that is activated by multiple stress stimuli. Most forms of stress-induced apoptosis engage the so-called mitochondrial apoptosis pathway. This pathway is controlled by the Bcl-2 protein family (Youle and Strasser, 2008). The Bcl-2 homology domain 3 (BH3)-only proteins are pro-apoptotic members of this family. BH3-only proteins are transcriptionally and post-translationally activated in neurons in response to stress (Bruijn et al., 2004; Ward et al., 2004), and induce apoptosis due to their ability to bind and neutralize anti-apoptotic Bcl-2 family proteins (Youle and Strasser, 2008). Activation of BH3-only proteins leads to the mitochondrial membrane insertion and oligomerization of Bax and Bak (Lovell et al., 2008; Tait and Green, 2010). The channels formed by these oligomers constitute release channels in the mitochondrial outer membrane, enabling the release of pro-apoptotic factors that trigger caspase-dependent and caspase-independent apoptosis. Bax was shown to accumulate in mitochondria in animal models of ALS (Guegan et al., 2001), and deletion of the *bax* gene in *mtSOD1* transgenic mice inhibited motoneuron death (Gould et

al., 2006). Recently it was shown that conditional, combined deletion of *bax* and *bak* potently delayed disease onset and progression in the *SOD1*^{G93A} mouse model of ALS (Reyes et al., 2010). BH3-only proteins responsible for the activation of Bax (and potentially Bak) in animal models of ALS were also recently identified. The BH3-only protein Bim was shown to be transcriptionally up-regulated in response to *mtSOD1* over expression, and deletion of *bim* protected against motoneuron loss in *mtSOD1* mice *in vivo* (Hetz et al., 2007). We showed recently that endoplasmic reticulum stress was able to activate the BH3-only protein Puma in motoneurons, and that deletion of *puma* protected motoneurons against cell death *in vitro* and in *mtSOD1* mice (Kieran et al., 2007).

Interestingly, several studies also suggest a non-cell autonomous mechanism of motoneuron loss in ALS (Ilieva et al., 2009). One possible explanation for a paracrine mechanism of motoneuron death is the release of pro-inflammatory or cell death-inducing cytokines from non-neuronal cells. Interleukin-1β (IL-1β) and Interferon-γ (IFNγ) have been implicated in ALS disease progression (Friedlander et al., 1997; Meissner et al., 2010; Wang et al., 2011). Motoneuron apoptosis in ALS was also shown to involve Fas ligand up-regulation and activation of Fas death receptors (Locatelli et al., 2007; Raoul et al., 2002). Release of pro-inflammatory cytokines may lead to death receptor and caspase-8 activation, which is able to directly activate executioner caspases such as caspase-3 (Locatelli et al., 2007; Raoul et al., 2002). However, in most cell types, this direct activation pathway is not sufficient to activate apoptosis, and an amplification loop is required for cell death execution that involves the BH3-only protein Bid, and hence engages the mitochondrial apoptosis pathway (Lovell et al., 2008; Luo et al., 1998). Indeed, increased expression of Bid in both neurons and astrocytes as well as increased levels of Bid cleavage were observed in symptomatic and late stage *SOD1*^{G93A} mouse spinal cords (Guegan et al., 2002). Interestingly, Bid was recently implicated in the production of pro-inflammatory cytokines in macrophages and microglia (Mayo et al., 2011) and was

reported to be involved in the activation of the transcription factor nuclear factor-κB (NF-κB) in response to stimulation of pattern recognition receptors in intestinal epithelial cells, independent of its direct apoptosis-regulating function (Yeretssian et al., 2011). Therefore, in the present study we sought to clarify the role of Bid in the context of neurodegeneration and neuroinflammation relevant to ALS.

MATERIALS AND METHODS

Animals

All experiments described in this study were performed under a license from the Department of Health and Children in Ireland (B100/3985) in accordance with the European Communities regulations 2010 (2010/63/EU). All procedures were previously approved by the Research Ethics Committee of the Royal College of Surgeons in Ireland. Transgenic SOD1 mice (*mus musculus*), IMSR: B6.Cg-Tg (*SOD1*^{*G93A}) 1Gur/J, with the incorporation of the G93A mutant form of human superoxide dismutase (*SOD1*), were purchased from The Jackson Laboratories (JAX, Bar Harbor, Maine, USA). *bid*$^{-/-}$ mice (*mus musculus*) were generated in the laboratory of Prof. Andreas Strasser, WEHI, Melbourne, Australia (Kaufmann et al., 2007). After weaning on postnatal day (PND) 28, all pups from litters of the same generation and colony were housed in groups of three to five per cage and maintained at 21±1 °C on a 12 h light/dark cycle, (07:00 h on; 19:00 h off) with *ad libitum* access to food and water.

Reagents and chemicals

Unless otherwise stated, chemicals were purchased from Sigma-Aldrich (Arklow, Ireland) or Merck Chemicals (Nottingham, UK). Cell culture media were purchased from Gibco-Life Technologies (Dun Laoghaire, Ireland). BI-6C9 was from Sigma-Aldrich (Pubchem: ID 24724408).

Primary motoneuron cultures

Primary motoneuron cultures (mixed cultures enriched for motoneurons) were prepared from wild-type and *bid* gene deficient (*bid*$^{-/-}$) E12 mouse embryos as described previously (Sebastia et al., 2009). Briefly, spinal cords ventral horns were dissected and tissue was removed and incubated for 10 min in 0.025% trypsin in Neurobasal media. Cells were

transferred into a 0.1 mg/ml DNase1 solution, and gently dissociated. Dissociated motoneurons were counted using a hemocytometer and seeded at a density of 10^5 cells/ml onto poly-D,L-ornithine/laminin-coated cell culture wells and maintained at 37°C and 5% CO_2. Motoneurons were maintained in complete Neurobasal media supplemented with 2% horse serum, 2% B27, GDNF (Promega, Cat#2781; 2 ng/mL), CNTF (R&D Systems, Cat#557-NT-10, 1 ng/mL), 100 U/mL penicillin and 100 μg/mL streptomycin. Medium was changed after 1, 3 and 6 days *in vitro* (DIV). Cells were cultured up to 14 days. Primary motoneuron cultures yielded mixed populations of cells with glial (ca. 40-60%), neuronal (ca. 30-40%) and non-neuronal morphology (5-10%); approximately 30-50% of the neuronal population were motoneurons positive for the motoneuron marker SMI-32 (Suppl. Fig. 3A-A'').

Isolation and culturing of primary astrocytes

Mixed glial cultures were prepared from the cortices of P2 *bid*$^{-/-}$ and wild-type mice. In brief, the cortices were dissected, the meninges removed before incubation in Minimum Essential Medium containing 0.025% trypsin and 0.1 mg/ml DNase I for 15 min at 37°C. The tissue was triturated mechanically in DMEM to dissociate cells, passed through a 40 μm nylon cell strainer (BD Falcon, Oxford, UK), and spun at 2,000 rpm for 5 minutes. The pellet was resuspended and plated in T75 flasks in DMEM-F12/L-Glutamine containing 1% Penicillin/Streptomycin and 10% Fetal Bovine Serum (Sigma-Aldrich). Cells were cultured for a minimum of 14 days before being passaged and cultured as astrocytes. For astrocyte-enriched cultures, astrocyte flasks were placed on the orbital shaker at 600 rpm to shake off microglia; remaining adherent cells were trypsinised and replated for the experiment at 5×10^5 cells per 6 well. Motoneuron media was used to generate astrocyte conditioned media (ACM) for motoneuron toxicity experiments (Neurobasal media with the above mentioned supplements).

Isolation of primary microglia

Microglia were isolated from mixed glial cultures (as above). Following two weeks of cell culture, the T75 flasks of confluent mixed glial cultures were shaken in DMEM/F12 at 600 rpm on a plate shaker for 8 h at room temperature. The supernatant was collected and spun down at 2,000 rpm for 5 minutes. The resuspended pellet was plated at a density of 1×10^5 cells/well of a 24 well plate. The isolated microglia were cultured for 2 days in well plates or Millicell-CM inserts (0.4 μm pore size, Fisher Scientific, Dublin, Ireland) in DMEM-F12/L-Glutamine, 10% Fetal Bovine Serum and 1% PenStrep (Sigma-Aldrich) before treatment.

Real-Time Quantitative PCR (qPCR)

RNA was extracted using Qiazol extraction and/or RLT-buffer lysis and RNeasy processing according to the manufacturer's guidelines (Qiagen, Sussex, UK). cDNA synthesis was performed based on equal amounts of RNA using the Superscript™ II Reverse Transcriptase (Invitrogen, California, USA). qPCR analysis was performed using the LightCycler (Roche Diagnostics, Basel, Switzerland) and the QuantiTech SYBR Green PCR kit (Qiagen) following manufacturer's recommendation and standard cycles and melting temperatures. The sense and antisense primers for glyceraldehyde-3-phosphate dehydrogenase (*gapdh*) were sense 5'-AAC TTT GGC ATT GTG GAA GG-3', antisense 5'-ACA CAT TGG GGG TAG GAA CA-3'; for *bid* 5'-TCC CCA GAG ACA TGG AGA AC-3' and 5'-GTC GTG TGG AAG ACA TCA CG-3'. RNA levels were normalized to *gapdh* mRNA expression and expressed as n-fold expression over control. For multiplex-genotyping of human SOD1G93A transgenic mice, primer sequences as described by the supplier (Jackson Laboratories, stock# 004435) were used. For *bid* genotyping, wild-type and deleted allele-specific primers using a three-primer strategy as described by (Kaufmann et al., 2007) were employed.

Western blotting and immunoprecipitation

Lumbar spinal cord lysates were assessed for Bid protein levels by Western blotting following dounce homogenization in RIPA-lysis buffer (Sigma-Aldrich). For Western-blot analyses of cell cultures, cells were rinsed with ice-cold PBS and lysed in RIPA lysis buffer containing protease inhibitor mix (Sigma-Aldrich). Protein content was determined using the BCA Micro Protein Assay kit (Pierce) and samples were supplemented with Laemmli sample buffer with 2-mercaptoethanol and denaturated at 95°C for 5 min, followed by electrophoresis and Western-blot by standard protocols. Blots were incubated with a rabbit polyclonal anti-Bid antibody (1:1000, AR-52, Enzo Life Sciences, Exeter, UK; Cat# ALX-210-007-R050, AntibodyRegistry (RIID): AB_2259218), a mouse monoclonal anti-GFAP (1:1000, Sigma-Aldrich Cat# G3893, RIID:AB_477010), a goat polyclonal anti-Iba-1 (1:500, Abcam, Cat# ab5076, RIID:AB_2224402), a rat monoclonal anti-CD11b (1:1000, Abcam Cat# ab8878, RIID:AB_306831), a rabbit polyclonal anti-MAP2 (1:1000, Santa Cruz Biotechnology, Cat# sc-20172, RIID:AB_2250101), rabbit anti-phosphorylated IKKα/β (1:500, 16A6, Cell Signaling Technology, Hitchin, UK, Cat# 2697L, RIID:AB_2291699) and anti-IKKα/β (1:500, H470, Santa Cruz Biotechnology, Inc., Cat# sc-7607, RIID:AB_675667), a rabbit polyclonal anti-PARP antibody (1:1000, Cell Signaling Technology Cat# 9542, RIID:AB_2160739), a rabbit polyclonal anti-caspase-3 (1:1000, Cell Signaling Technology Cat# 9662S, RIID:AB_10694681), mouse monoclonal α-tubulin (Sigma-Aldrich, Cat# T6199, RIID:AB_477583), and β-Actin (clone AC-40, Sigma-Aldrich, Cat# A3853, RIID:AB_262137) antibodies (both 1:5000), a rabbit monoclonal phospho-Ser536p65 (clone 93H1, CST, Cat# 3033S, RRID:AB_331284), a rabbit monoclonal pan-p65 (clone D12E12, Cell Signaling Technology Cat# 8242P, RRID:AB_10859369). A goat polyclonal anti-Bid (AF860, R&D Systems, Cat# AF860, RIID:AB_2065622) or normal goat-IgG (Santa Cruz) were used for immunoprecipitation analyses and captured by protein A/G agarose bead slurry (Santa Cruz Biotechnology) following standard protocols. Here, following Laemmli-buffer lysis and electrophoreses a rabbit polyclonal anti-IKKγ

(1:500, Abcam Cat# ab77750, RIID:AB_156633) was used for immunodetection. Membranes were incubated with the species-specific peroxidase-labeled secondary antibodies diluted 1:1000-10,000 (Pierce, Northumberland, UK). Bands were detected using Immobilon Western Chemiluminscent HRP Substrate (Fisher Scientific) and imaged using a FujiFilm LAS-3000 imaging system (Fuji, Sheffield, UK). Western-blot optical densities were determined using standard procedures using ImageJ (NIH; imagej.nih.gov/ij)

Immunofluorescence and microscopy

Primary cultured neurons were exposed to propidium iodide (2 μg/ml, Sigma Cat#P4864) or trypan-blue solution (0.4%, Life Technologies Cat#15250061) prior to fixation in 3% paraformaldehyde (PFA, Polyscience, Eppelheim, Germany, Cat#18814-20) diluted in cytoskeletal buffer (CB-buffer; 10 mM PIPES pH 6.8, 300 mM NaCl, 10 mM EGTA, 10mM glucose, 10mM $MgCl_2$) for 12 minutes. Fixed cells were permeabilized by incubation with ice-cold PBS containing 0.1 % (w/v) Triton X-100 and blocked with 0.3 % (w/v) Triton X-100 and 5 % (v/v) horse serum in PBS. They were incubated overnight in primary antibody at 4 °C diluted in PBS / 0.3 % (w/v) Triton X-100 / 3 % (v/v) horse serum. We used a mouse monoclonal anti-neurofilament heavy polypeptide (SMI32, 1:1000, Covance, Maidenhead, UK, Cat# SMI-32P-100, RIID:AB_10719742), as well as the above mentioned p-IKK and GFAP-antibodies. Secondary Alexa-Fluor-488 or -568 coupled secondary antibody (1:500, Life Technologies) was incubated for 1 hour at room temperature following extensive washes. Cell counts of trypan-blue/PI and SMI-32 labeling were performed manually by two experienced experimenters (blinded). Photomicrographs were taken using a SPOT RT SE 6 Camera (Diagnostic Instruments, Sterling Heights, MI, USA) on an Eclipse TE 300 inverted microscope (Nikon, Kingston upon Thames, UK) with Mercury-arc excitation and appropriate filter settings.

Reporter assays

Astrocyte cultures ~~following~~ were transfected using Roche X-tremeGENE HP reagent (Roche, Dublin, Ireland) using standard protocols with a mixture of pGL4.32[luc2P/NF-κB-RE/Hygro] vector (NF-κB-RE, firefly reporter vector, Promega, Southampton, UK, Cat#E8491, GenBank:EU581860.1) and a Renilla-luciferase expressing construct under constitutive thymidine-kinase promoter control (phRL-TK-luc, Promega, Cat#E6241) at a ratio of 14:1 for normalisation. Cultures were washed extensively and transferred back into their respective conditioned media for cytokine treatments on the subsequent day. Cells were lysed in passive-lysis buffer (PLB, Promega) following the indicated periods of time of cytokine or lipopolysaccharide (LPS) treatment in their respective conditioned media, and assayed by Dual-luciferase assay (Promega, Cat#E19160) according to the manufacturer's specifications.

Statistical analysis

Statistical analyses were performed using PASW statistics 17 software (SPSS, IBM, Dublin, Ireland) or GraphPad Prism (GraphPad Software Inc., La Jolla, CA, USA). Significance was determined using two-tailed student t-test, or Mann-Whitney U test for non-parametric data. For multiple comparisons, one-/two-way ANOVA followed by Holm-Sidak or Tukeys post-hoc test was used. For non-parametric data, Kruskal-Wallis H test test, or Friedmann test for matched data were used, and Dunn's *post-hoc* test. Grubbs test was used to test for outliers. All data are represented as mean ± range or mean ± SEM. p values ≤ 0.05 were considered to be significantly different and marked by an asterisk.

RESULTS

Bid protein levels increase in the SOD1^{G93A} mouse during disease progression

Bid is constitutively expressed in many central neurons including motoneurons (Krajewska et al., 2002), and its activation during apoptosis involves proteolytic cleavage rather than transcriptional activation (Li et al., 1998; Luo et al., 1998). However, there ~~has~~ was also ~~been~~ a report demonstrating increased full-length Bid protein levels during disease progression in *SOD1^{G93A}* mice (Guegan et al., 2002). We therefore started our investigation to re-examine the role of Bid regulation during motoneuron degeneration. Western blotting analysis of glial-acidic fibrillary protein (GFAP) and Bid protein levels in lumbar spinal cord lysates of *SOD1^{G93A}* mice showed moderately elevated full-length Bid protein levels in 'presymptomatic' postnatal day (PND) 50 mice, or symptomatic PND 90 mice, and a pronounced, significant up-regulation in late stage PND 120 *SOD1^{G93A}* mice when compared to their non-transgenic counterparts (Fig. 1A,B; d120: transgenic increased by +139.9% ± 84.62%). Concurrently, GFAP protein levels, likely including GFAP splice variants (Kamphuis et al., 2012), increased significantly *in vivo* (Fig.1A, d120: transgenic increased by +685.1% ± 51.39%). We also detected reduced immunoreactivity of the motoneuron marker SMI-32, and moderately up-regulated levels of the microglial marker Iba-1 in the lumbar spinal cord of these transgenic animals (Fig.1A). A lack of commercially available antibodies that reliably recognize Bid protein levels by immunohistochemistry (i.e. that did not show significant non-specific staining in *bid*-deficient mice; Suppl. fig. 2A-C,F) prevented us from exploring which cell types expressed Bid during disease progression. However, previous reports suggested significant Bid immunostaining in both neuronal and non-neuronal cells (Franz et al., 2002; Guegan et al., 2002). In addition, we failed to collect significant evidence for Bid cleavage in the spinal cord of *SOD1^{G93A}* transgenic animals between day50 and day120 (Suppl. Fig. 1A-D), or astrocytes exposed to a pro-inflammatory environment (Suppl. Fig. 1E).

Bid is expressed in primary astrocyte and microglia cultures

To explore whether Bid is also expressed in non-neuronal cells, we investigated Bid protein levels by quantitative PCR and Western blotting in murine mixed motoneuron, primary astrocyte as well as microglial cultures that were generated using established isolation protocols. While motoneuron cultures exhibited moderate *bid* mRNA (Fig. 2A) and protein levels, we noted elevated Bid levels in cultured astrocytes, and an even stronger enrichment of Bid protein in microglia-enriched cell cultures (astrocyte over motoneuron lysates: increase of 279.5% ± 23.4%, microglia over motoneuron lysate: increase of 799.3% ± 43.5%; Fig. 2B&C). These results suggested that Bid may play a role in the regulation of neuroinflammation and astrocyte activation.

Bid is activated in cultured astrocytes exposed to pro-inflammatory stimuli

Neuroinflammation is a common hallmark of many neurodegenerative disorders including ALS. To determine whether pro-inflammatory stimuli modulate Bid protein levels, we treated dissociated astrocyte-enriched cultures with the TLR-agonist lipopolysaccharide (LPS 100 ng/ml, 24 h). Western-blot analysis or immunofluorescence labeling revealed a remarkable induction of Bid-protein levels in astrocytes treated with LPS (Fig. 3A,B, Suppl. fig. 2A,C,D). Spinal cord tissue of ALS mice were shown to have elevated levels of secreted interferon-γ (IFNγ, Wang et al., 2011) and interleukin-1β (IL-1β, Meissner et al., 2010) during pre-symptomatic, symptomatic and end stages. Treatment of mixed motoneuron cultures or astrocyte-enriched cultures with IFNγ and IL-1β resulted in an increase of Bid protein to levels comparable to those induced by LPS (Figs. 3C-F, Suppl. fig. 2B), meanwhile, GFAP protein levels largely remained unaltered (Fig. 3E). Bid up-regulation occurred in the absence of astrocyte cell death (data not shown), and elevated levels of activated, cleaved caspase-3 or of caspase-3-specific cleavage products of PARP-1 were not detectable, demonstrating the absence of an activation of the extrinsic or intrinsic apoptosis pathway in astrocytes (Fig. 3G&H). In contrast to our observations in

astrocytes or mixed glial or mixed motoneuron cultures (Fig. 3C,I,J, Suppl. Fig. 2D&E), exposure of microglia-enriched cultures to LPS or IFNγ/IL-1β did not alter Bid protein levels (Fig. 3K-M, Suppl. Fig. 2D), suggesting that Bid is specifically induced in astrocyte cultures in response to TLR agonists and pro-inflammatory cytokines. This induction was similarly observed in Western-blot and immunofluorescence analyses in purified and mixed motoneuron and glial cultures, with the caveat of a high non-specific background immunofluorescence present in the *bid*-deficient astrocytes and tissue seen using three distinct full-length Bid-specific antibodies (Suppl. Fig. 2A-C,F).

Bid negatively modulates nuclear factor-κB activity in cultured astrocytes

Activation of astrocytes during ALS pathogenesis has been attributed both neuroprotective and neurotoxic activities. Bid ~~has~~ was recently ~~been~~ implicated in the control of inflammation in intestinal epithelial cells by regulating the activation of the pro-inflammatory transcription factor nuclear factor-κB (NF-κB) through functional interaction with the IKK-complex (Yeretssian et al., 2011). We hence immunoprecipitated LPS-stimulated astrocyte lysates using Bid anti-serum. In activated cells, Bid markedly interacted with the NF-κB essential modulator (NEMO), a critical component of the IKK-complex (Fig. 4A). Notably, we observed increased levels of IKK-kinase (IKKβ) phosphorylation in astrocyte cultures exposed to IFNγ and IL-1β, and this was reduced by prior co-application of the Bid-inhibitor BI-6C9 (Fig. 4B). Concomitantly, we also observed increased levels of IKK-kinase (IKKβ) and p65 phosphorylation in astrocyte cultures exposed to IFNγ and IL-1β or LPS, which were not observed in *bid*-deficient astrocytes (Fig. 4B-D). We next investigated whether Bid was implicated in pro-inflammatory cytokine-induced NF-κB activation in astrocytes. We monitored the activity of the NF-κB transcription factor in reporter gene assays in wild-type and *bid*-deficient astrocyte cultures. Exposure of NF-κB-reporter-gene transfected astrocyte cultures to IL-1β and IFNγ demonstrated that pro-inflammatory cytokine-induced stimulation of NF-κB was

significantly reduced by co-application of the small molecule Bid inhibitor BI-6C9 (Fig. 4E). Likewise, *bid*-deficiency significantly reduced pro-inflammatory cytokine-induced NF-κB activity (Fig. 4F). Furthermore, prolonged exposure of astrocyte cultures to a pro-inflammatory environment through application of IFNγ and IL-1β for three days to one week resulted in a substantial rise in the levels of the NF-κB target gene cyclooxygenase-II (COX-II), which was ablated in *bid*-deficient astrocyte cultures that were free from notable CD11b immunoreactivity (Fig. 4G-H).

bid-deficiency protects against non-cell autonomous motoneuron death

To explore whether *bid*-deficiency had functional consequences in the context of non-cell autonomous motoneuron death, we treated either wild-type or *bid*-deficient primary astrocyte cultures with LPS or vehicle (saline). After wash-out of LPS, we collected the respective astrocyte conditioned media (ACM) and transferred them to wild-type motoneuron cultures (Fig. 5A). Motoneuron survival was evaluated by counting SMI-32 positive neurons that were also negative for propidium iodide and trypan-blue following twenty-four hours of incubation (Fig. 5B). Following assessment of mixed motoneuron cultures using the additional motoneuron marker HB9 (Arber et al., 1999, Suppl. Fig. 3B-B''), we determined that all SMI-32 positive cells in our cultures were NeuN as well as HB9-positive motoneurons (Suppl. Fig. 3A'',B'') and that SMI-32 represents a stringent motoneuron marker in mixed motoneuron cultures (Carriedo et al., 1996). A significant decrease in surviving, SMI-32 positive cells was noted following incubation of motoneurons with wild-type ACM medium (decreased by 26 ± 19 percent), but not following their exposure to medium generated by *bid*-deficient astrocytes (increased by 6 ± 15 percent; Fig. 5C). Similarly, we observed a significant increase in survival when motoneurons were exposed to the glial-conditioned media (GCM) from *bid*-deficient astrocytes co-cultured with wild-type astrocytes, when compared to wild-type astrocytes co-cultured with wild-type microglia (increased by 1.26 ± 0.224 fold, Fig. 5D,E).

DISCUSSION

Mutant SOD1 (*mtSOD1)* was suggested to cause motoneuron degeneration by impairing axonal transport, facilitating protein aggregate production, and sequestering heat-shock proteins and anti-apoptotic proteins (Pasinelli and Brown, 2006). The toxicity of *mtSOD1* was also shown to involve non-cell autonomous cell death pathways (Ilieva et al., 2009; Lee et al., 2012). Astrocytes expressing *mtSOD1* were shown to be a primary source of the pro-inflammatory cytokine IFNγ which may directly or indirectly kill neighbouring motoneurons (Siegmund et al., 2005; Aebischer et al., 2011; Wang et al., 2011). Increased expression of pro-inflammatory cytokines, including IL-1β, IFNγ and IL-6, were a common pathological hallmark seen in both transgenic *SOD1*^{G93A} mice and ALS patients (Li et al., 2000; Lasiene and Yamanaka, 2011; Wang et al., 2011). Direct administration of IFNγ, or IFNγ derived from SOD1 mutant astrocytes, induced motoneuron degeneration *in vitro* in a selective and dose-dependent manner (Siegmund et al., 2005; Beers et al., 2006; Boillee et al., 2006; Aebischer et al., 2011). On the other hand, neuroinflammatory processes may also stimulate tissue regeneration and exert beneficial effects during neurodegeneration (Morganti-Kossman et al., 1997; Wyss-Coray et al., 2002; Zhao et al., 2006; Lalancette-Hébert, 2007). Our data indicate that Bid is induced during neuroinflammation in ALS. We observed a marked induction of Bid protein at the symptomatic stage in *SOD1*^{G93A} mice that occurred in parallel with a markedly upregulated GFAP and moderately elevated levels of the microglial marker Iba-1. In line with our results, Bid expression was ~~has been~~ previously shown to be markedly elevated in both neuronal and non-neuronal cells in the *SOD1*^{G93A} mouse (Guegan et al., 2002), and to correlate with disease severity and progression in ALS patients, reaching a maximum at end stage (Guegan et al., 2002). In experiments using enriched motoneuron, astrocyte and microglia cultures, we demonstrated that Bid was preferentially expressed in astroglia and microglia. We further found it was strongly induced in astrocytes upon exposure to pro-inflammatory stimuli, but

was not induced in stimulated microglia. Bid was previously shown to be required for death receptor-induced apoptosis in numerous cell types, and couples death receptor signaling to the mitochondrial apoptosis pathway (Li et al., 1998; Luo et al., 1998; Gross et al., 1999). While IFNγ and IL-1β themselves do not engage death receptors, it was shown that IFNγ-activated microglia induce the expression of the pro-inflammatory cytokine TNF-α and hence may indirectly activate the extrinsic apoptosis pathway. IFNγ may also co-operate with TNF-α to induce oxidative stress (Hanisch, 2002; Mir et al., 2009). Additionally, increased Fas ligand expression and activation were identified as key mediators of apoptosis in motoneurons during disease progression in the *SOD1*^{G93A} mouse (Raoul et al., 2002; Locatelli et al., 2007). Our data suggest that Bid, in addition to a role in death receptor-induced apoptosis of motoneurons, may play a role in non-cell autonomous motoneuron death by controlling NF-κB activity in astroglia.

Our studies revealed that *bid*-deficiency or the application of a small molecule Bid inhibitor significantly reduced LPS-, IFNγ and IL-1β-induced NF-κB activity in astrocytes. Suppression of pathologically elevated NF-κB activity in astroglia rather than inhibition of death receptor signaling may indeed underlie the protection afforded by astrocyte *bid*-deficiency described in this study. The effects of Bid on NF-κB activation in astrocytes occurred in the absence of caspase-3 activation, suggesting a non-apoptotic role of Bid in this context. Interestingly, suppression of pathologically elevated NF-κB activity has repeatedly been linked to improved outcome in CNS injury (Brambilla et al., 2009), sciatic nerve injury and ALS (Fu et al., 2010; Otsmane et al., 2013). A 'non-apoptotic' function of Bid may be in accordance with previous studies that demonstrated a direct influence of Bid on NF-κB activity (Luo et al., 2010; Yeretssian et al., 2011). The latter study suggested a biological link between the NOD1 (nucleotide-binding oligomerization domain containing 1) signalosome and Bid in intestinal epithelial cells, evidenced by co-immunoprecipitation between Bid protein, components of the inflammasome and the IκB kinase (IKK)-complex,

and a marked deficiency of *bid* deficient cells to phosphorylate the canonical IKK target protein, IκBα. In accordance with these studies, we found activated astrocytes expressed a protein complex comprising IKKγ and Bid. However, recently other results ~~have shown~~ showed that Bid may not be essential for NOD signaling in other cell types (Nachbur et al., 2012), suggesting that Bid effects on NF-κB activation are highly context- and tissue-dependent.

The pro-inflammatory potency of glial Bid expression may be partially mediated by cyclooxygenase-2, a NF-κB-induced mediator of prostaglandin E2 output (Roshak et al., 1996; Kaltschmidt et al., 2002). Notably, COX-II enzyme is potently up-regulated in the ALS-diseased human spinal cord (Yiangou et al., 2006). These results are also in agreement with a study showing a non-apoptotic, Bid-dependent activation of COX-II in fibroblasts exposed to alkylating agents (Luo et al., 2010). Recently, it was found that *bid*-deficient macrophages and microglia also exhibited an impaired ability of pro-inflammatory cytokine production (Mayo et al., 2011). It is therefore conceivable that Bid plays a key role in the supply of pro-inflammatory cytokines from both microglia and astroglia (Nagai et al., 2007), thereby amplifying inflammation (Yamanaka et al., 2008). In line with a non-apoptotic role of Bid, we failed to detect the pro-apoptotic, cleaved form of Bid, tBid, by Western blot analyses during disease progression in *SOD1*^{G93A} mice using three separate antibodies detecting either full-length (AF-860 (R&D Systems), AR-52 (Enzo Life Sciences); Fig 1A, Suppl. Fig. 1A-C,E), or cleaved Bid (AB10002, EMD Millipore; Suppl. Fig. 1D). However tBid is a short-lived protein, which may be difficult to detect.

In summary, our study highlights a new, non-apoptotic role for astrocytic Bid in the control of neuroinflammation, and suggests that Bid activation may contribute to non-cell autonomous motoneuron degeneration in ALS.

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FIGURE CAPTIONS

Figure 1. Increased Bid protein levels during disease progression in SOD1^{G93A} mice. **(A)** Western blot analysis of full length Bid, GFAP, SMI-32 and Iba-1 protein levels in wild-type and transgenic *SOD1^{G93A}* mice at PND120 during the symptomatic stage of ALS disease progression (marks highlight GFAP splice variants). **(B)** Quantification of Bid protein levels by Western-blot analyses of non-transgenic (*wt*) and transgenic *SOD1^{G93A}* (*tg*) spinal cords pre-symptomatically (PND50), at disease onset (PND90) and during disease (PND120). Data are normalized to respective wild-type as well as β-Actin loading and represented as mean ± range (PND50(n=4/4), PND90(6/6), PND(21/22),*p=0.0462 (PND120) Mann-Whitney U test).

Figure 2. Elevated Bid-protein levels in astroglial cells. (A,B) Dissociated murine motoneurons cultures (DIV7), astrocyte-enriched cultures and microglia-enriched cultures were lysed in RLT (A, n=3) or RIPA-buffer (B) and mRNA reverse transcribed and subjected to real-time PCR analysis using *bid*- and *gapdh* specific primers, while proteins were separated by gel-electrophoresis. Western-blots were exposed to a rabbit polyclonal Bid-antibody, as well as antibodies detecting the neuronal marker protein MAP2, the astroglial marker GFAP, and the microglial marker Iba-1. **(C)** Quantification of Bid protein levels compared to motoneuron content was derived from five Western-blot experiments (A,C: mean ± range).

Figure 3. Increased Bid levels in astroglial cultures following pro-inflammatory treatment. Mouse astroglial (**A,B**) or mixed glial cultures (composed from 50/50% microglial and astroglial cells; **I,J**) were treated with 100 ng/ml LPS for twenty-four hours, lysed and subjected to Western-blot analyses. (**C-F**) Mouse motoneuron (C,D) or astroglial (E,F) cultures were treated for twenty-four hours with IFNγ (500 U/ml) and IL-1β (50 ng/ml) or vehicle in cell culture media and lysates were subjected to gel-electrophoresis and Western-blot analyses using the antibodies as indicated. (**K-M)** Microglia-enriched cultures

were cultured for one week and exposed to 100 ng/ml LPS (K,L) or the cytokine mix (M) as delineated above and subjected to gel-electrophoresis and Western-blot analyses. Quantification of Bid protein levels was derived by optical density analysis from Western-blot experiments and normalized to loading control (B, n=3; D, n=1, F, n=2; J, n=2; L, n=4; mean ± range).

Figure 4. Bid-protein functionally interacts with essential components of the IKK-signalosome and contributes to NF-κB target-gene induction. (A) Murine astrocytes were treated for 24 h with the TLR-agonist LPS (100 ng/ml), cells were lysed and immunoprecipitated using rabbit-derived anti-Bid antiserum. A notable immunoreactivity against NEMO was noted in the immunoprecipitated samples and a concurrently decreased NEMO immunoreactivity in the supernatant. Bid immunoreactivity was detected using mouse anti-Bid antibodies in the immunoprecipitates. (h.c., heavy-chain) **(B)** Cultured wild-type *(wt)* or *bid*-deficient astrocytes were exposed to either vehicle, LPS, or a combination of the Bid-inhibitor BI-6C9 (10 μM, 30 min pre-incubation) and LPS for 24 h, as indicated. Western-blot membranes derived from the RIPA cell lysates were exposed to phosphorylation-specific IKKα/β and p65 antibodies, and pan-IKKα/β, pan-p65 or loading control antibodies following membrane stripping. **(C)** The optical density data from three Western-blots of LPS-treated (1-7 days) wild-type *(wt)* or *bid*-deficient astrocytes directed at phosphorylated IKKβ was determined and normalized to total IKKβ levels (mean ± range). **(D)** wild-type or *bid*-deficient astrocytes were exposed for 24 h to LPS, fixed and immuno-labeled against phosphorylated-IKKα/β (red) and GFAP and S100b (green), as indicated. **(E)** Astrocyte cultures, transfected with the κB-dependent reporter gene vector, were treated with either IL-1β or IL-1β (50 ng/ml) + IFNγ (500 U/ml), with or without the Bid-inhibitor BI-6C9 (100 μM) or respective vehicle, as indicated. The bar graphs represents κB-dependent relative firefly activity (n=3-8 from two pooled experiments, one outlier

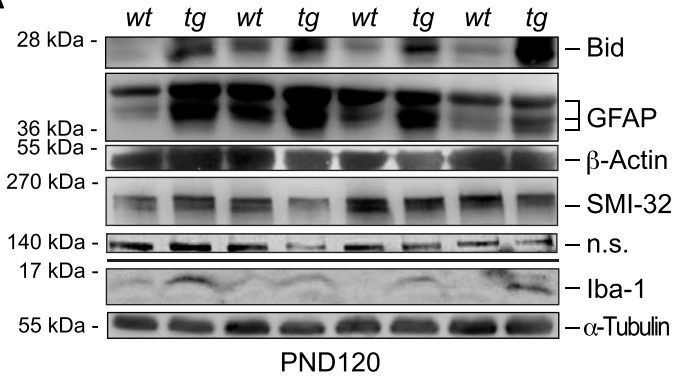
removed, data represents mean ± SEM). **(F)** Mixed wild-type or *bid*-deficient astroglial cultures were transfected with κB-response element luciferase reporter vectors and subsequently treated with IFNγ (500 U/ml) and/or IL-1β (50 ng/ml) for 24 h as indicated. A significantly decreased level of NF-κB activity in Bid-deficient cells was noted (n=10-16 wells from three pooled experiments, 1 outlier removed, data represents mean ± SEM). **(G,H)** Astrocyte-cultures were grown to confluency, repeatedly stimulated with the pro-inflammatory cytokine mix every two days for a total of 3 days (G), or one week (H) and lysed. Following gel-electrophoresis and Western-blotting, blots were exposed to antibodies as indicated. The dotted line indicates intervening lanes have been spliced out. **(I)** Experiments G&H were repeated and the quantified optical densities from a total of n=4 biological replicates was normalized to loading control and graphed (mean ± range).

Figure 5. **Bid-protein deficiency ablates the potency of activated astrocytes to kill wild-type motoneurons. (A)** Diagram describing the collection of activated astrocyte-conditioned media and their application to motoneuron cultures. **(B,C)** Wild-type or *bid*-deficient astrocytes were treated with 100 ng/ml LPS for 24 hours in astrocyte media. Cells were washed extensively and motoneuron media was conditioned on thus activated astrocytes for 48 h without further cell stimulus. The media collected from these cells (ACM) was applied to wild-type motoneurons for 24 h, cells were exposed to trypan-blue solution, fixed and SMI-32 (inset, green) and PI (inset, red) labeled. A potent decrease of SMI-32 positive motoneuron density to 74% was recorded following wild-type ACM exposure, but motoneurons were potently protected when exposed to *bid*$^{-/-}$-derived ACM (*, $p \leq 0.05$ for wild-type vs *bid*$^{-/-}$, two-way ANOVA, pooled from 2 experiments from 4 litters, n=8-10 from 4 biological replicates, data represents mean ± SEM). Assessment of trypan-blue positive cells yielded significantly reduced cell death in *bid*-deficient cells versus wild-type (p=0.0009 for wild-type vs *bid*$^{-/-}$, two-way ANOVA, data not shown). Scale

bars 50 μm, 10 μm in insets. **(D,E)** wild-type or *bid*-deficient astrocytes were co-cultured with wild-type microglia as depicted (D) and these mixed cultures were treated with 100 ng/ml LPS for 24 h in astrocyte media. Cells were washed extensively and motoneuron media was conditioned on these activated astrocytes for 48 h without further cell stimulus. The media collected from these cells (GCM) was applied to wild-type motoneurons for 48 h, cells were exposed to trypan-blue solution, fixed and SMI-32 and PI labeled. A significant protection was noted of following exposure to $bid^{-/-}$-derived GCM (*, $p \leq 0.05$, one-sided t-test, n=6-7 wells, pooled from 2 biologically separate experiments, data represents mean ± SEM).

Figure 1

A


wt tg wt tg wt tg wt tg
28 kDa -
– Bid
36 kDa -
55 kDa -
– GFAP
– β-Actin
270 kDa -
– SMI-32
140 kDa -
– n.s.
17 kDa -
– Iba-1
55 kDa -
– α-Tubulin
PND120

B

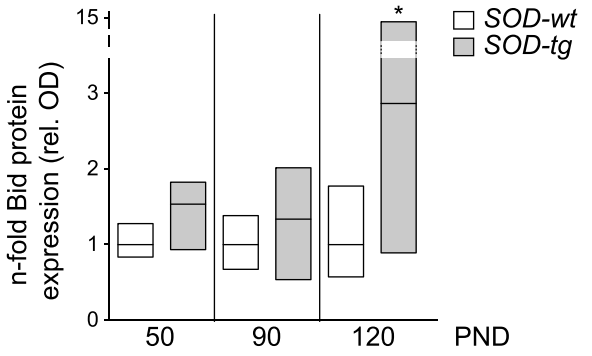

n-fold Bid protein expression (rel. OD)
15
3
2
1
0
*
SOD-wt
SOD-tg
50
90
120
PND

Figure 2

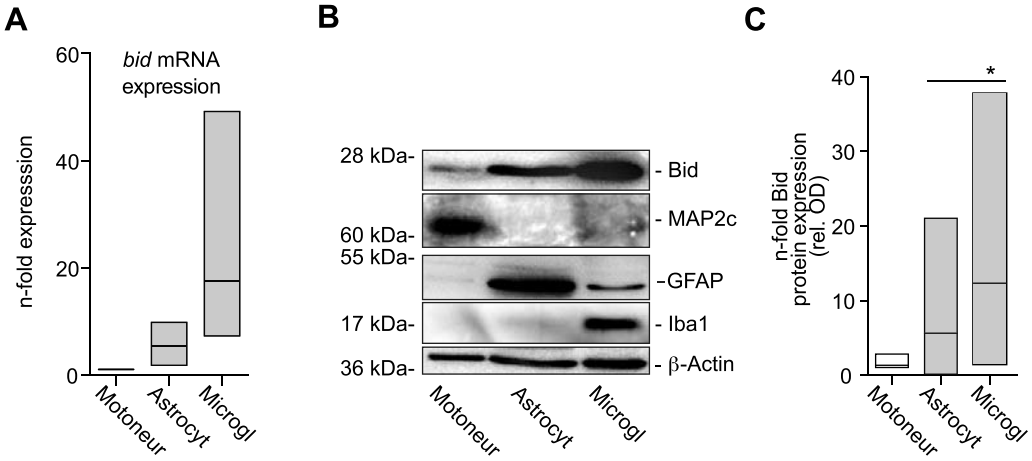

A
bid mRNA expression
n-fold expresssion
60
40
20
0
Motoneur
Astrocyt
Microgl
B
28 kDa-
- Bid
60 kDa-
- MAP2c
55 kDa-
-GFAP
17 kDa-
- Iba1
36 kDa-
- β-Actin
Motoneur
Astrocyt
Microgl
C
n-fold Bid protein expression (rel. OD)
40
30
20
10
0
*
Motoneur
Astrocyt
Microgl

Figure 3

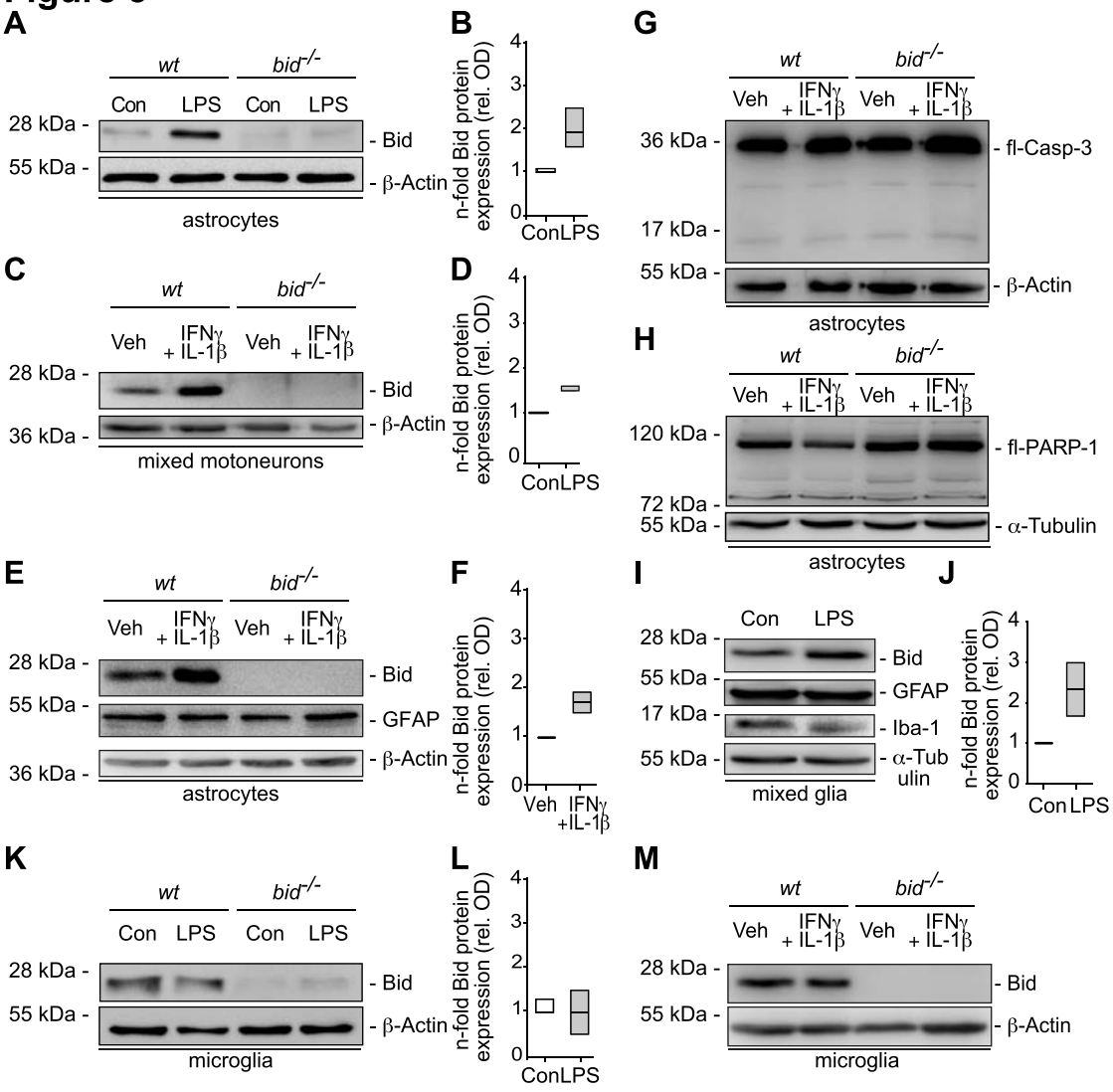

A
wt
bid-/-
Con
LPS
Con
LPS
28 kDa -
55 kDa -
- Bid
- β-Actin
astrocytes
B
n-fold Bid protein expression (rel. OD)
Con
LPS
C
wt
bid-/-
Veh
IFNγ + IL-1β
Veh
IFNγ + IL-1β
28 kDa -
36 kDa -
- Bid
- β-Actin
mixed motoneurons
D
n-fold Bid protein expression (rel. OD)
Con
LPS
E
wt
bid-/-
Veh
IFNγ + IL-1β
Veh
IFNγ + IL-1β
28 kDa -
55 kDa -
36 kDa -
- Bid
- GFAP
- β-Actin
astrocytes
F
n-fold Bid protein expression (rel. OD)
Veh
IFNγ +IL-1β
G
wt
bid-/-
Veh
IFNγ + IL-1β
Veh
IFNγ + IL-1β
36 kDa -
17 kDa -
55 kDa -
- fl-Casp-3
- β-Actin
astrocytes
H
wt
bid-/-
Veh
IFNγ + IL-1β
Veh
IFNγ + IL-1β
120 kDa -
72 kDa -
55 kDa -
- fl-PARP-1
- α-Tubulin
astrocytes
I
Con
LPS
28 kDa -
55 kDa -
17 kDa -
55 kDa -
- Bid
- GFAP
- Iba-1
- α-Tubulin
mixed glia
J
n-fold Bid protein expression (rel. OD)
Con
LPS
K
wt
bid-/-
Con
LPS
Con
LPS
28 kDa -
55 kDa -
- Bid
- β-Actin
microglia
L
n-fold Bid protein expression (rel. OD)
Con
LPS
M
wt
bid-/-
Veh
IFNγ + IL-1β
Veh
IFNγ + IL-1β
28 kDa -
55 kDa -
- Bid
- β-Actin
microglia

Figure 4

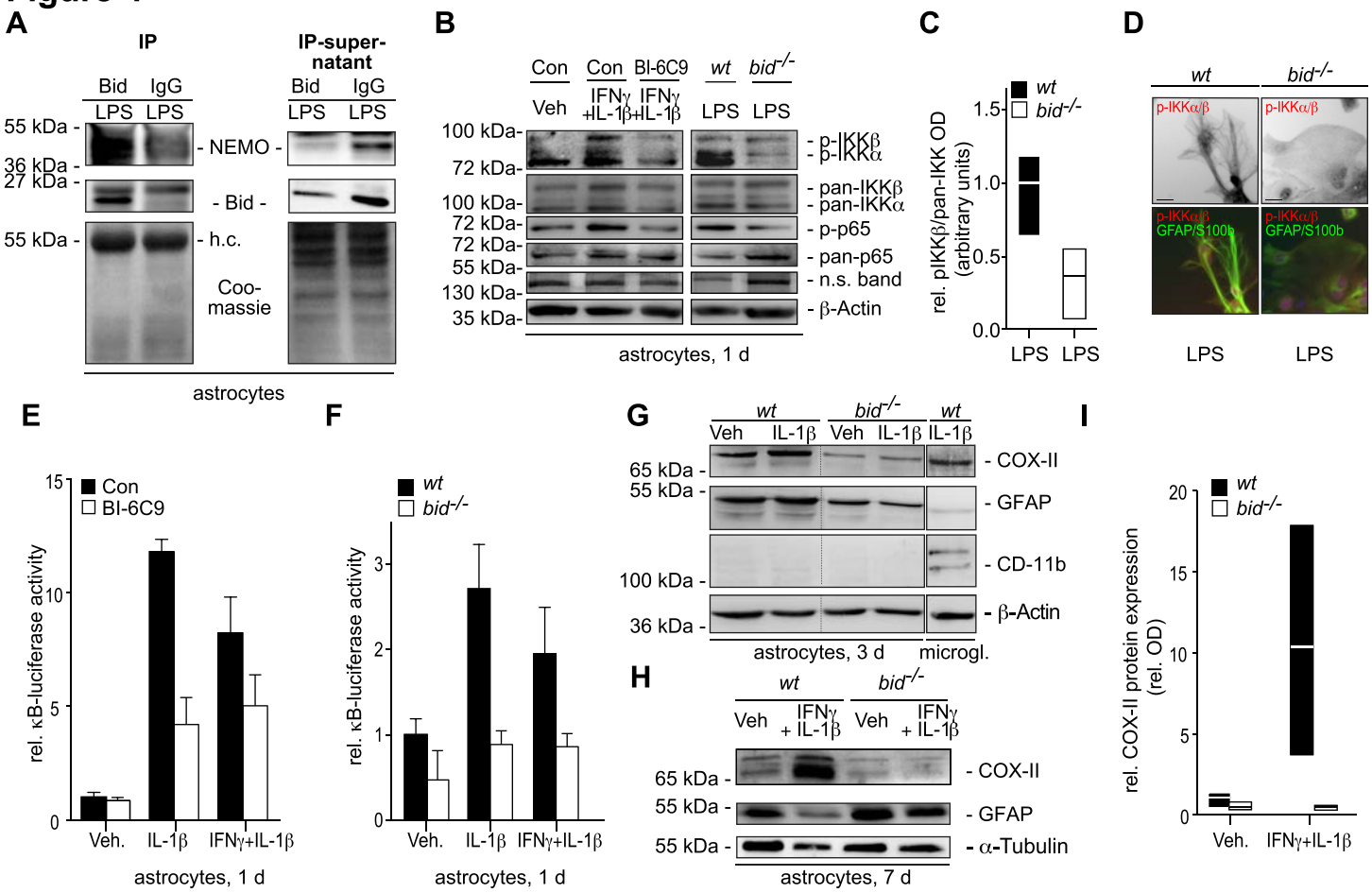
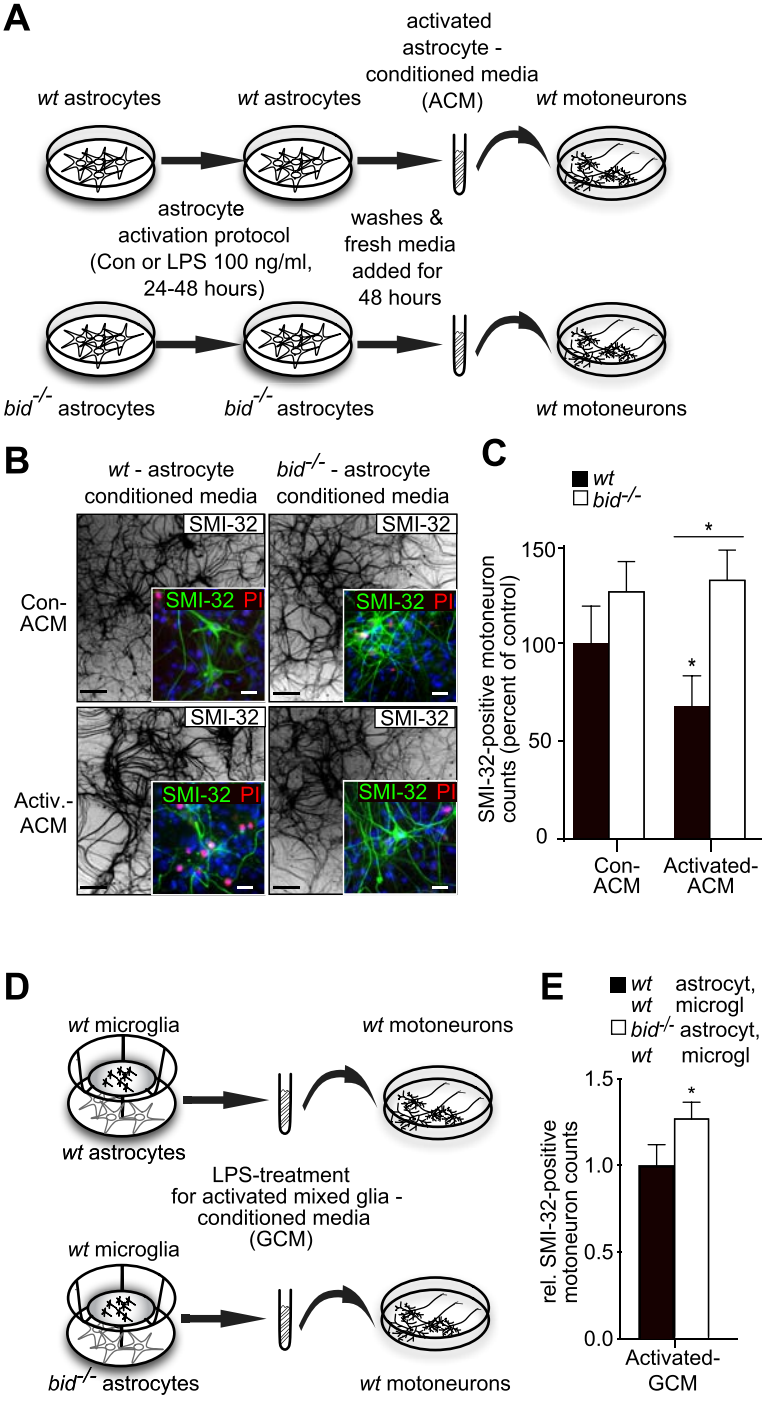
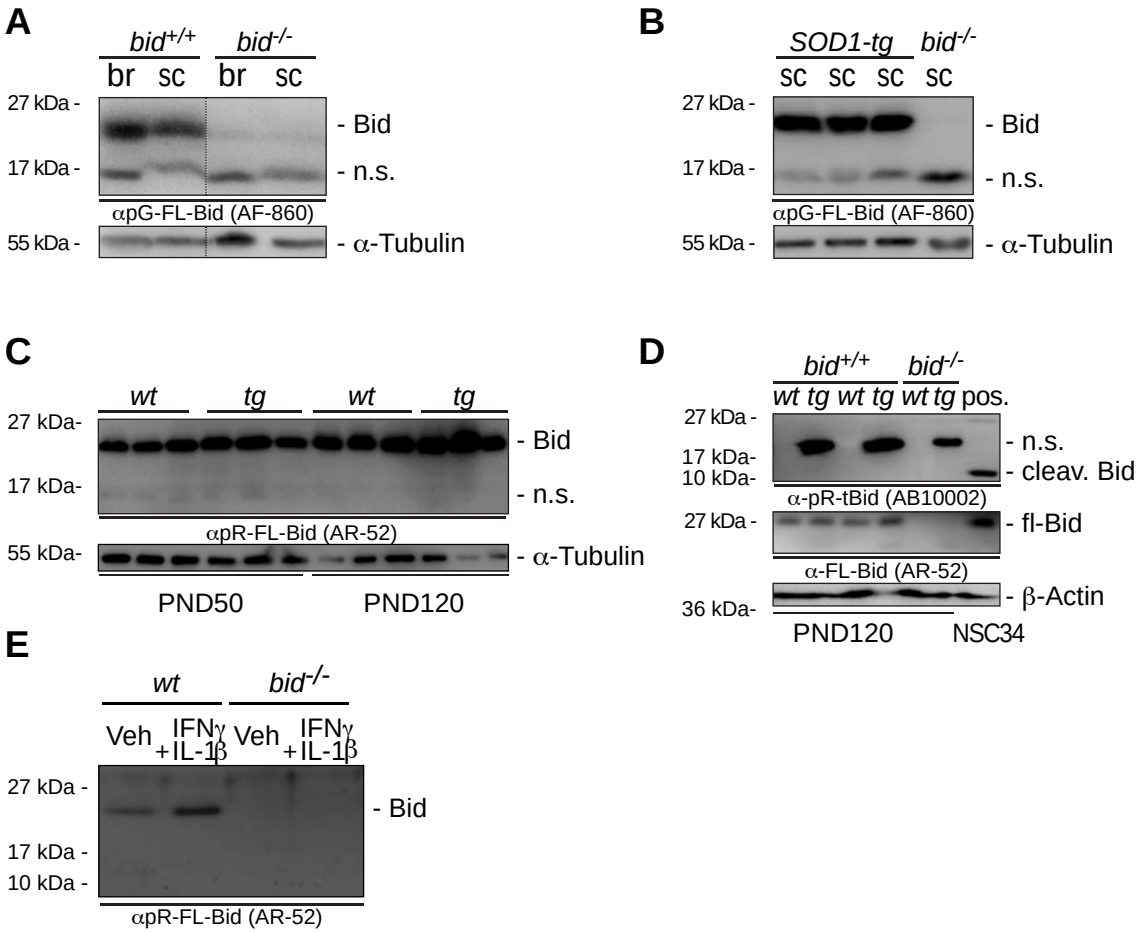

A
IP
IP-super-natant
Bid IgG
LPS LPS
55 kDa -
36 kDa -
27 kDa -
- NEMO -
- Bid -
55 kDa -
- h.c.
Coo-massie
astrocytes
B
Con Con BI-6C9 wt bid-/-
Veh IFNγ +IL-1β IFNγ+IL-1β LPS LPS
100 kDa- - p-IKKβ
72 kDa- - p-IKKα
100 kDa- - pan-IKKβ
72 kDa- - pan-IKKα
72 kDa- - p-p65
55 kDa- - pan-p65
130 kDa- - n.s. band
35 kDa- - β-Actin
astrocytes, 1 d
C
wt
bid-/-
rel. pIKKβ/pan-IKK OD (arbitrary units)
1.5
1.0
0.5
0.0
LPS LPS
D
wt bid-/-
p-IKKα/β
p-IKKα/β GFAP/S100b
LPS LPS
E
Con
BI-6C9
rel. κB-luciferase activity
15
10
5
0
Veh. IL-1β IFNγ+IL-1β
astrocytes, 1 d
F
wt
bid-/-
rel. κB-luciferase activity
3
2
1
0
Veh. IL-1β IFNγ+IL-1β
astrocytes, 1 d
G
wt bid-/- wt
Veh IL-1β Veh IL-1β IL-1β
65 kDa - - COX-II
55 kDa - - GFAP
100 kDa - - CD-11b
36 kDa - - β-Actin
astrocytes, 3 d microgl.
H
wt bid-/-
Veh IFNγ + IL-1β Veh IFNγ + IL-1β
65 kDa - - COX-II
55 kDa - - GFAP
55 kDa - - α-Tubulin
astrocytes, 7 d
I
wt
bid-/-
rel. COX-II protein expression (rel. OD)
20
15
10
5
0
Veh. IFNγ+IL-1β

Figure 5

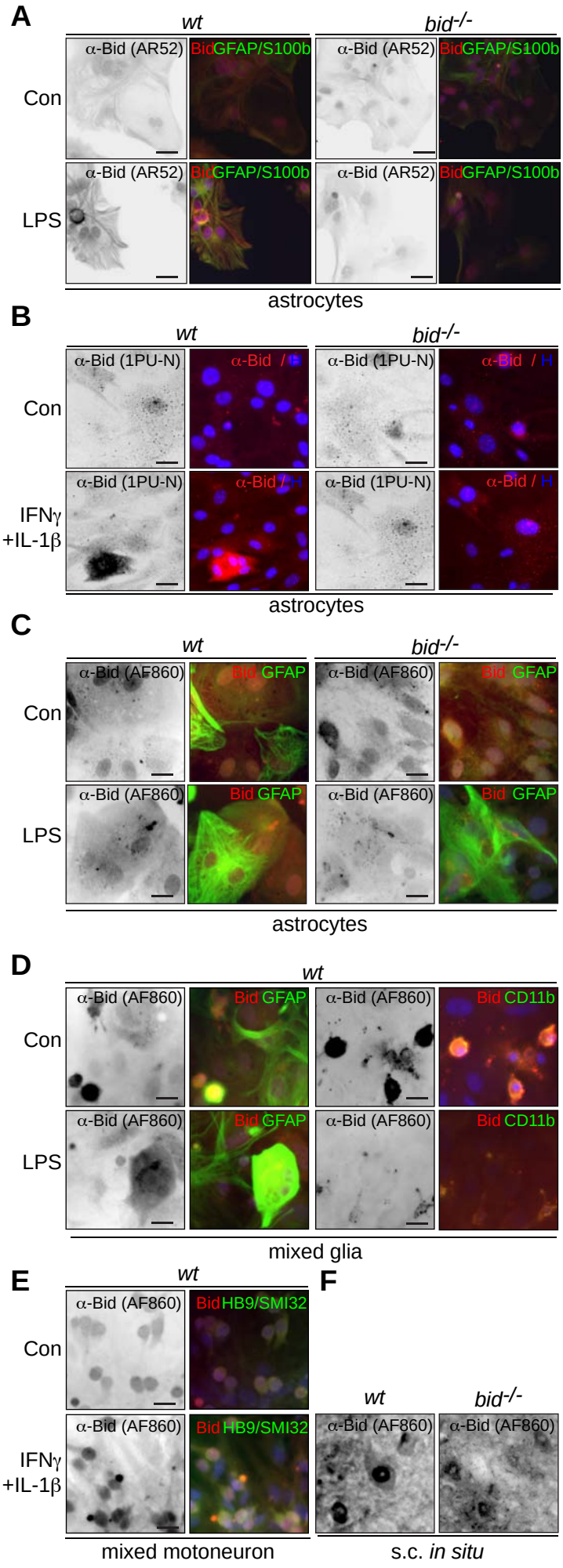

A
activated astrocyte - conditioned media (ACM)
wt astrocytes
wt astrocytes
wt motoneurons
astrocyte activation protocol (Con or LPS 100 ng/ml, 24-48 hours)
washes & fresh media added for 48 hours
bid-/- astrocytes
bid-/- astrocytes
wt motoneurons
B
wt - astrocyte conditioned media
bid-/- - astrocyte conditioned media
Con-ACM
Activ.-ACM
SMI-32
SMI-32 PI
C
wt
bid-/-
SMI-32-positive motoneuron counts (percent of control)
150
100
50
0
*
*
Con-ACM
Activated-ACM
D
wt microglia
wt motoneurons
wt astrocytes
LPS-treatment for activated mixed glia - conditioned media (GCM)
wt microglia
bid-/- astrocytes
wt motoneurons
E
wt astrocyt, wt microgl
bid-/- astrocyt, wt microgl
rel. SMI-32-positive motoneuron counts
1.5
1.0
0.5
0.0
*
Activated-GCM

SUPPLEMENTARY FIGURE 1



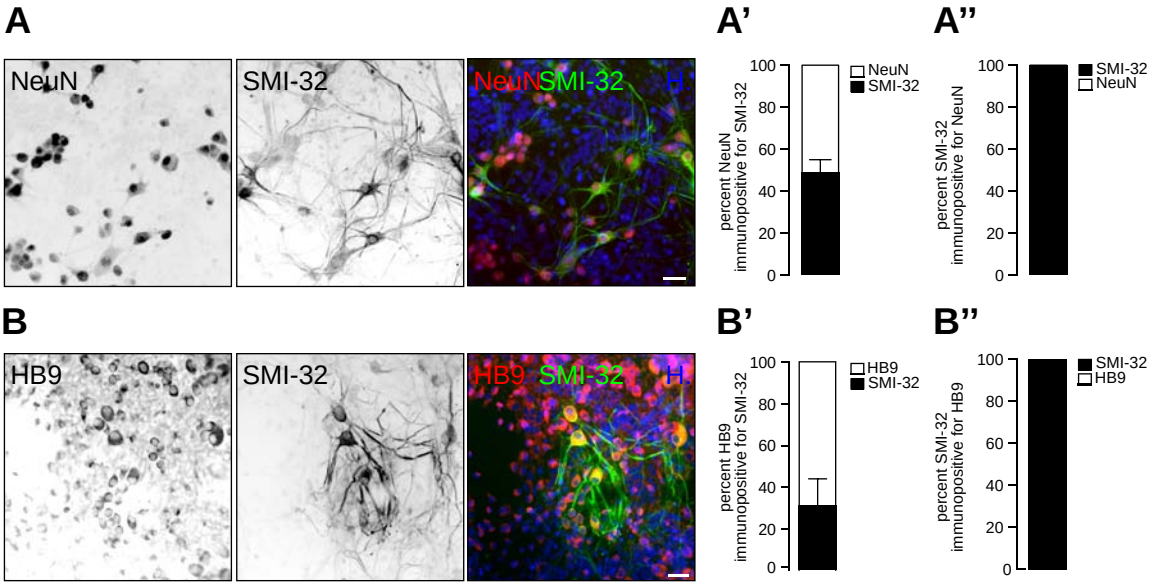
Supplementary figure 1 caption. *Lack of evidence for Bid cleavage products in inflammatory stimulus exposed neural cells or spinal cord tissues following comparison between wild-type and bid-deficient lysates.* **(A)** *Wild-type* as well as *bid-deficient* brain (br) and spinal cord (sc) samples were collected in RIPA buffer and probed for Bid protein using a polyclonal goat (pG) anti-Bid antibody raised against the full-length protein (1:1000; R and D Systems Cat# AF860, AntibodyRegistry (RIID):AB_2065622) by Western blot analyses using standard procedures. Anti-Bid immunoreactivity is detected at an apparent molecular weight of approximately 22 kDa. Note immunoreactivity of a non-specific band (n.s.) at approximately 13-17 kDa in *bid*$^{+/+}$, but likewise in lysates from *bid*-deficient brain and spinal cord samples. A mouse anti-α-tubulin antibody was used as loading control mouse monoclonal α-tubulin (1:5000, Sigma-Aldrich, Cat# T6199, RIID:AB_477583). The dotted line denotes intervening bands have been spliced out. **(B)** End stage *SOD1*^{G93A} *transgenic* (*tg*) spinal cord (sc) samples and a *bid*$^{-/-}$ control sample were sampled for Bid protein analysis by Western blot analysis. End stage SOD-tg animals exhibited minimal immunoreactivity below 20 kDa, while a *bid*-deficient sample exhibited marked reactivity at an identical molecular weight. This anti-Bid antibody immunoreactivity was considered non-specific in subsequent analyses. **(C)** Postnatal day (PND) 50 and PND120-derived spinal cord lysates from *wild-type* as well as *SOD1-transgenic* mice as highlighted were exposed anti-Bid (AR-52), raised against the full length protein (1:1000, AR-52, Enzo Life Sciences, Exeter, UK; Cat# ALX-210-007-R050, RIID: AB_2259218) or α-tubulin antibodies following electrophoresis and Western-blot, respectively. Note the minimal immunoreactivity below 17 kDa visible across all individuals examined. **(D)** PND120 lysates of *wt* and *tg* spinal cord tisse tissue as well as staurosporine-treated (300 nM, 6 hours) NSC34 cells used as positive control for Bid protein cleavage (pos.), were subjected to 15 % polyacrylamide gel electrophoresis and subsequent Western blot followed by exposure to a rabbit anti-mouse Bid cleavage site (59/60) specific polyclonal antibody (upper panel, 1:500, EMD Millipore Cat# AB10002, RRID:AB_570963), a rabbit anti-mouse Bid (middle panel) and anti-mouse β-Actin (lower panel, clone AC-40, 1:5000, Sigma-Aldrich, Cat# A3853, RIID:AB_262137). Please note the strong immunoreactivity of a non-specific (n.s.) band that is also present in the *bid*-deficient *tg* tissue at around 17 kDa, while no immunoreactivity was noted around the apparent molecular weight of the expected 10-15 kDa of the caspase-8 generated cleavage product (cleav.) of Bid as present in the positive control. **(E)** Expanded view of the anti-Bid blot generated from mixed motoneuron cultures following cytokine exposures as depicted in Figure 3C. Prolonged exposure times did not yield any notable Bid-cleavage product-derived immunoreactivity in a range between ten and twenty kDa.

SUPPLEMENTARY FIGURE 2



Supplementary figure 2 caption. *Testing antibodies directed at mouse full-length Bid against Bid-deficient cells and tissue* **(A-C)** *wt*- and *bid*$^{-/-}$ astrocyte cultures were exposed to lipopolysaccharide for 24 h, fixed in 3% paraformaldehyde solution for 12 minutes at 37ºC, and subjected to immunofluorescence analyses using the standard protocol. The cells were exposed to the antibodies followed by standard exposure to fluorescently-labelled secondary antibodies, a rabbit polyclonal anti-Bid antibody (A,red, 1:50, AR-52, Enzo Life Sciences, Exeter, UK; Cat# ALX-210-007-R050, AntibodyRegistry (RIID): AB_2259218), mouse anti-GFAP/S100b antibodies (A-D, 1:500, both green, Sigma Cat# G-A-5, RRID:AB_2314539, mouse S100b, 1:500, Sigma, Cat# S2532, RRID:AB_477499), a rabbit polyclonal anti-Bid antibody (B,red,1:100, Cat# AP06621PU-N, RRID:AB_1611121, a kind sample of Acris Antikörper GmbH) and a goat polyclonal anti-Bid (**C-F**, AF860, R&D Systems, Cat# AF860, RIID:AB_2065622). For the generation of mixed glial cells, astrocytes were mixed to 50/50 % with microglial cultures, seeded and fixed following the treatments as indicated. These cultures were additionally exposed to a rat monoclonal anti-CD11b for the labelling of microglia (**D**, 1:500, Abcam Cat# ab8878, RIID:AB_306831). Consider the significant background staining observed using all three mouse Bid-specific antibodies in *bid*-deficient, (non-)treated controls (third column, A-D) that overall matches - in extent as well as in distribution - the immunofluorescence observed in *wt*-cells (first column, A-D). Also note elevated anti-Bid immunofluorescence in some but not all astrocytes treated with cytokine or LPS (A-C) and the strong staining for anti-Bid in the microglia compared to the astrocytes in the mixed cultures (D), which match the result of higher Bid baseline expression in microglia than astrocytes obtained in the Western-blot analyses (Figure 2B&C). The significant background immunofluorescence in *bid*$^{-/-}$ cells suggests a cautious interpretation. A rabbit anti-Hb9 (green, 1:4000, Abcam Cat# ab79541, RIID:AB_1603361) and a mouse anti-SMI32 (1:1000, Covance, Cat#SMI-32P, RIID:AB_10719742), both motoneuron markers were used in (**E**) to mark these cells in the mixed motoneuron cultures. Please note the moderate elevation of anti-Bid-immunofluorescence in the cytokine-treated motoneurons, as well as the astrocytes beneath the motoneurons in these mixed cultures. Spinal cord (s.c.) motor neurons *in situ* were labelled in **(F)** using the goat-anti-Bid antibody. Note the slightly lower, but noteable fluorescence generated following application of this antibody to the *bid*-deficient spinal cord tissue compared to the *wt* tissue that hindered unambiguous assessment of Bid expression patterns using this antibody in our spinal cord tissues. Treatment regimen concentrations matched the concentrations outlined in the main manuscript. Hoechst-nuclear labelling is present in all multicolour images in blue. Scale bar 20 µm.

SUPPLEMENTARY FIGURE 3



Supplementary figure 3 caption. *SMI-32 immunolabelling represents a stringent method to highlight motoneurons in mixed motoneuron cultures.* **(A)** Mixed Motoneuron cultures were maintained for ten days in their cell culture media as described. The primary motoneurons were fixed in 3% para-formaldehyde solution diluted in cytoskeletal buffer (CB-buffer; 10 mM PIPES pH 6.8, 300 mM NaCl, 10 mM EGTA, 10 mM glucose, 10 mM $MgCl_2$) for 12 minutes at 37ºC, and subjected to immunofluorescence analyses using the standard protocol. The cells were exposed to the following antibodies, a mouse anti-SMI-32 (anti-Neurofilament H Non-Phosphorylated, 1:1000, green, Covance, Inc. Cat# SMI-32P-100, RIID:AB_10719742, a motoneuron marker) and a rabbit anti-NeuN (anti-NEUronal nuclei, 1:1000, red, EMD Millipore Cat# ABN78, RIID:AB_10807945, labelling all neuronal nuclei). **(A',A'')** Three wells were immunolabelled as above and the fraction of NeuN-positive cells that were also immunopositive for SMI-32 was determined. Note that all cells found positive for SMI-32 were exclusively NeuN immunopositive (A'') and that a substantial fraction of the neuronal cells in culture expressed the SMI-32 antigen (46%, A'). **(B)** Primary Motoneuron cultures were maintained and fixed as above and exposed to the SMI-32 antibody here combined with a rabbit anti-HB9 /HLXB9 specific antibody (motor neuron and pancreas homeobox 1, 1:4000, red, Abcam Cat# ab79541, RIID:AB_1603361, a motoneuron marker). **(B',B'')** Three wells were immunolabelled as above and the fraction of HB9-positive cells that were also immunopositive for SMI-32 was determined. Note that all cells found to express SMI-32 were also labelled by HB9 (B''), but that in turn only a fraction of cells immunopositive for HB9 were co-labelled by SMI-32 (29%, B'). Hoechst-nuclear labelling is present in all multicolour images in blue. Scale bar 20 µm.